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#9
Harris
June 1, 01
09-386,850

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of
Rosinski-Chupin et al.

Group art Unit : 1811
Examiner : S. Marshall

Serial N° : 08/476,120
Filed : June 7, 1995

For : Peptides and polypeptides derived from the submaxillary gland of the rat.... for detection or therapeutic purposes

DECLARATION UNDER RULE 132

Hon. Commissioner of Patents and Trademarks
WASHINGTON D.C. 20231
Sir :

I, Isabelle ROSINSKI-CHUPIN, residing at 37, avenue Pierre Grenier, 78220 VIROFLAY (France) ;
having the degree of "Doctorat es Sciences"
working for INSTITUT PASTEUR
declare as follows :

I am one of the inventors of the present application and I am aware of the fact that claims 27-28 are rejected under 35 USC 112 first paragraph, as failing to provide an enabling disclosure.

The specification of the present patent application presented the high degree of accumulation of the mRNA coding for SMR1 in the submaxillary gland (SMG) of the rat in response to androgens (page 12) : the level of accumulation of mRNA coding for SMR1 in the SMG of female Wistar rats was about 1,000 to 3,000 times lower than that in males (lines 8-11) ; in the castrated males, the quantity of

mRNA coding for SMR1 was reduced 10 to 20 fold, and the administration of testosterone to these males restored the amount of mRNA (lines 11-15).
In the light of the results showing the high degree of induction of the SMR1 gene by androgens (lines 29-30), I could infer, at the time of the invention, that the accumulation of the claimed peptides is correlated to the androgen activity and that, consequently, the claimed peptides can be useful as markers of the androgen activity.
More particularly, the claimed peptides can be very useful as biological markers for evaluating the androgen or anti-androgen activity of new drugs to be tested in preclinical trials.
Thus, it clearly appears that the use of the claimed peptides was suggested to anyone skilled in the art when reading the present patent application.

The following experiments, in which I closely took part, are consistent with the results described in the patent application and confirm the correlation between the claimed peptides and the androgen activity, and the use of the claimed peptides for evaluating the androgen activity.
In accordance with the testosterone-dependent accumulation of the SMR1 mRNAs in SMG, we showed that mRNA of SMR1 protein was accumulated at a high level in the acini of SMG of male, whereas mRNA of SMR1 protein could barely be detected in female (section A).
We confirmed that SMR1 encoding gene expression was correlated with acinar differentiation on the one hand, and with androgen increase during puberty in males on the other hand (section B).
Then we used antibodies prepared according to the standard procedures, as suggested in the specification of the patent application (page 5, lines 16-30), for assaying SMR1-related peptides and as expected, we showed that biosynthesis of these peptides strongly increases after puberty and that the level of these peptides in SMG is much higher in males than in females (section C).
Hypophysectomy and gonadectomy experiments confirmed that the expression of the claimed peptides is significantly correlated to the androgen activity (section D).

A. **The localization of mRNA of SMR1 protein by *in situ* hybridization reveals sexual differences in acinar cells of rat submandibular gland (SMG) :**

Androgen-dependent sexual differences in the granular convoluted tubules of mouse and rate submandibular glands have been extensively reported.

We showed that SMR1 mRNAs are synthesized in the acinar cells of the SMG and demonstrated a clear difference in SMR1 mRNA levels in male and female.

Materials and methods

- Tissue preparation.

Ten-week-old male and female Wistar rats (Iffa Credo, Lyon France) were sacrified by CO_2 suffocation ; submandibular and sublingual glands were rapidly removed, rinsed in PBS (0.008 M Na_2HPO_4, 0.002 M KH_2PO_4 0.14 M NaCl, 0.0035 M KCl, pH 7.4), cut in two or three pieces, and immersed overnight in 4 % paraformaldehyde (Merck ; Darmstadt, Germany) in PBS at 4°C with constant shaking. In a control experiment, rats were anesthesized by IP injection of Imalgene 500 (Ketamine 150 mg/kg) (Rhône-Merieux, France) and perfused via jugular veins with 0.85 % NaCl and then with 4 % paraformaldehyde in PBS. Salivary glands were then removed and immersed overnight in 4 % paraformaldehyde in PBS at 4°C as before. In all cases tissue sections were rinsed in PBS, then in saline solution at 4°C, dehydrated in graded ethanol, and embedded in paraffin (Paraplast +), (Sherwood Medical ; Athy, Ireland). Seven-µm thick sections were obtained with a microtome and mounted on gelatin-coated slides.

After deparaffinization, slides were post-fixed in fresh 4 % paraformaldehyde in PBS for 20 minutes at room temperature (RT), rinsed with PBS, treated with proteinase K 20 µg/ml for 7.5 minutes, and fixed again for 5 minutes. At this point, they were acetylated with 0.25 % acetic anhydride in 0.1 M ethanolamine for 10 minutes and dehydrated through a graded series of ethanol.

- Plasmids.

The SMR1 cDNA obtained by polymerase chain reaction and cloned in pSP64 vector (Promega France ; Charbonnières, France) as previously described (Rosinski-Chupin I., and Rougeon F., *one amino acid change in rat SMR1 polypeptide induces a 1kDa difference in its apparent molecular mass determined by electrophoretic analysis*, FEBS Letters, 1990, 267, 147-149), was recloned between the EcoRI and PstI sites of the pSP65 vector, using the internal PstI site brought in by the 3'PCR primer. The plasmid is named SMR1pSP622.

PcS1-1 was obtained by subcloning the EcoRI-HindIII fragment of SMR1pSP622 between the EcoRI and HindIII sites of pcDNAII (Invitrogen Co. ; Oxon, U.K.). The insert therefore corresponds to the nearly complete SMR1 cDNA, except for the first 21 5' bases of the RNA and the last 43 3' bases plus the poly-A tail.

pUC42 is a GRP cDNA cloned in the laboratory during the screening of our SMG cDNA library and corresponds to the $pCRP_3$ cDNA (described by Heinrich and Habener, *genes encoding proteins with highly homologous contiguous repeat sequences are highly expressed in the serous cells of the rat submandibular gland. J. Biol. Chem. 1987;262:5262*). PcG-1 was obtained by subcloning the Sau3A-EcoRI fragment of pUC42 between the BamHI and the EcoRI sites of pcDNAII. This fragment corresponds to the sequence encoding the transition region, the repeats region, and most of the carboxy terminal part of the protein.

- *In situ* hybridization.

Digoxigenin-labeled RNA probes were synthesized with a digoxigenin-labeling kit (Boehringer Mannheim, France ; Meylan, France) according to the manufacturer's instructions and were submitted to mild hydrolysis by heating at 60°C for 5-15 minutes in 100 mM Na_2CO_3 before neutralization and ethanol precipitation. *In situ* hybridization experiments were performed overnight at 48°C with ~ 20-200 ng of probe in 25 µl of hybridization buffer (50 % formamide, 0.3 M NaCl, 20 mM Tris-HCl, pH 7.4 5 mM EDTA, 10 mM NaH_2PO_4, 10 % dextran sulfate, 1 x Denhardt's, 0.5 mg/ml yeast RNA) for each slide. After hybridization, the slides were washed in 5 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M Na-citrate), 10 mM dithiothreitol (DTT) at 42°C for 30 minutes and in 2 x SSC, 50 % formamide, 80 mM DTT at 60°C for 20 minutes. Then, they were treated with 20 µg/ml RNAse A in RNAse buffer (0.4 M NaCl, 5 mM EDTA, 10 mM Tris-HCl, pH 7.4) for 30 minutes at 37°C and washed in (a) RNAse buffer, (b) 2 x SSC, and (c) 0.1 x SSC, each time for 15 minutes at 37°C.

All subsequent treatments were at RT. Slides were dipped first for 45 minutes into 0.5 % blocking reagent (Boehringer Mannheim, France), in Buffer 1(150 mM NaCl, 100 mM Tris-HCl, pH 7.5) and then for 45 minutes in 1 % bovine serum albumin (fraction V), (Sigma, St Louis, MO), 0.3 % TritonX-100 in the same buffer. After a 2-hour incubation with anti-digoxigenin F(ab) fragments labeled with alkaline phosphatase (Boehringer Mannheim, France) diluted 1:2500 in Buffer 1, the slides were rinsed five times for 10 minutes each in Buffer 1 and once for 5 minutes in Buffer2 (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris, pH 9.5). Alkaline phosphatase activity was detected by reaction with nitroblue tetrazolium chloride 0.33 mg/ml (Sigma), plus 5-bromo-4-chloro-3-indolylphosphatase 0.16 mg/ml (sigma) in Buffer 2 for 1.5 hour in the dark. The reaction was stopped by dipping the slides in water. Slides were counterstained with Harris' hematoxylin (Rhône-Poulenc ; Villers St Paul, France) and mounted in Eukitt (Kindler ; Freiburg, Germany).

Results

Hybridization of 10-week-old male rat SMG sections with digoxigenin-labeled anti-sense SMR1 RNA resulted in a strong signal localized in acinar cells. This signal was RNA specific, as it was completely abolished by pre-treatment of the section with RNAse A. In addition, no signal was obtained with sense RNA probes, demonstrating that this signal was specific for SMR1 mRNA. Endogeneous alkaline phosphatase, which can be detected on myoepithelial cells directly after deparaffinization was not seen under our conditions of *in situ* hybridization.

All acini were positive for SMR1 expression and no specific signal was detected in intercalated, GCT, and striated ducts. In acinar cells, the signal was strongest around the nucleus and near the basal membrane and was more diffuse, forming a fine network, in the apical cytoplasm.

In contrast, in sections of SMGs of female rats, no signal could be detected except for very few acinar cells. This result was independent of the procedure for tissue fixation (direct immersion in the fixative or perfusion of the whole rat by fixative).

No signal was detected in the adjacent sublingual gland in male and in female sections.

As positive control for tissue preparation, we studied GRP expression, for which no sexual difference has been described. GRPs are strongly expressed in the acini. As expected, no sex-linked difference was detected in GRP expression.

Conclusion

The level of accumulation of the SMR1 mRNAs in rat SMG was shown to be dependent on testosterone. In accordance with this, our *in situ* hybridization results reveal a clear sexual difference in the acini of rat SMG : whereas the acini are strongly positive in males, SMR1 mRNAs are barely detectable in the SMG of females.

B. The expression of the gene encoding SMR1 protein correlated with acinar differentiation and androgen activity

We investigated the relationships between the expression of the gene encoding SMR1 protein and the differentiation of acinar cells on the one hand and the hormonal regulation on the other hand.

Materials and methods

Wistar rats were purchased from Iffa Credo (Lyon, France). Male and female rats (of different ages : newborn, 5, 12 and 18 days old, 4, 6, 10, 11 weeks old) were euthanized with carbon dioxide and submandibular glands were dissected and were either immediatly frozen in liquid nitrogen and stored at -80°C prior RNA isolation or fixed in 4 % paraformaldehyde in PBS overnight at 4°C. RNA was prepared from pooled submandibular glands as described.

RNase protection experiment

- Probes.

A 234 bp DNA fragment (nucleotides 174 to 407 of the cDNA disclosed on page 9 of the patent specification) was generated by PCR and cloned in the pcDNA II vector (Invitrogen Corp.) (pca1var). A 124 bp fragment corresponding to the 5'end of the actin cDNA (SmaI digested) was subcloned in pCDNAII (pcat5') and used as an internal standard in RNase protection experiments.

- RNase protection.

RNase protection experiments were performed with the RNase 1 enzyme (Promega) according to manufacturer's instructions. Briefly, antisense RNA were generated by *in vitro* transcription in presence of (α-32P)UTP at 160 Ci/mmol from the T7 or SP6 promotor. The two probes (200000 cpm ; 0.6ng for each probe) were mixed and hybridized with a mixture of rat submandibular total RNA and yeast tRNA (total quantity = 20 µg) during 16 hours, at 45°C in 30 µl of hybridization buffer (80 % formamide, 40 mM pipes pH 6.4, 0.4 M sodium acetate, pH 7 and 1 mM EDTA). Non-hybridized RNA were digested with 5-10 U RNAse 1 in 300 µl digestion buffer (10 mM Tris pH 7.5, 5 mM EDTA, 200 mM sodium-acetate, pH 7) for one hour at 37°C. Digestions were stopped by addition of SDS (final concentration 0.1 %) and tRNA (60 µg/ml). After ethanol precipitation, the samples were loaded on a sequencing gel.

Quantification : signals were quantified using the Phosphorimager system (Molecular Dynamics).

- RNA blot analysis

Total RNA was electrophoresed in an agarose/formaldehyde gel, transferred to a nylon membrane (Hybond N, Amersham) and hybridized with probes labeled by the random priming method. The GRP probe was a GRP cDNA cloned in the laboratory during the screening of our SMG cDNA library and corresponding to

the pCRP3cDNA described by Heinrich and Habener. The actin probe was a rat b-actin cDNA cloned in the laboratory. Hybridization signals were quantified with the phosphorimager system.

Results

1. Expression of GRP mRNAs as a marker for acinar differentiation in the rat SMG during post-natal development.

To obtain a model curve of mRNA accumulation kinetics for a typical acinar gene, we semi-quantitatively studied GRP mRNA relative levels, during the first 11 post-natal weeks. This was done by Northern blot analysis of individual preparations of rat SMG mRNA at different ages, using a GRP cDNA probe and quantification of the signals by scanning with phosphorimager. In our conditions of electrophoretic resolution, the two GRP RNA bands, which differ in size by about 100 nucleotides were not separated and were quantified together.

To correct for differences of exposure between the different blots, a panel of three RNA preparations was included in every blot and was used to homogenize the results between the different blots. A good reproductibility was observed between the results of different Northern blot experiments for a same sample. The blots were also hybridized with an β-actin cDNA probe to control for the presence of variations in transfer efficiencies or RNA quantities.

Quantification of β-actin mRNA signal revealed that no strong differences in actin mRNA quantities could be detected between individuals at the same age, whatever their sex. However, β-actin mRNA levels, relative to the same quantity of total RNA, were shown to be higher during the first two weeks of age and decrease during post-natal development (between birth and 11 weeks). A plateau is reached at about 3-4 weeks, despite some variations may be noted in six weeks old animals. No significant sex-linked difference was noticeable at any age.

The results of GRP mRNA quantifications are given in figure 1. Low levels of GRP mRNAs were detected as soon as birth. A high increase in GRP mRNA levels occurs between days 0 and 5. A plateau of GRP mRNA expression is reached at 4 weeks, at a value about 20-25 times higher than for day 5. No significative sex-linked difference ($p>0.05$) can be noted except at day 12 where values in males were seen as slightly higher than in females and in 11 weeks old animals where values in females are about 1.2 fold higher than in males. No variation in GRP mRNA levels was seen between animals at the same age.

Variations of GRP mRNA accumulation during post-natal development of rat SMG

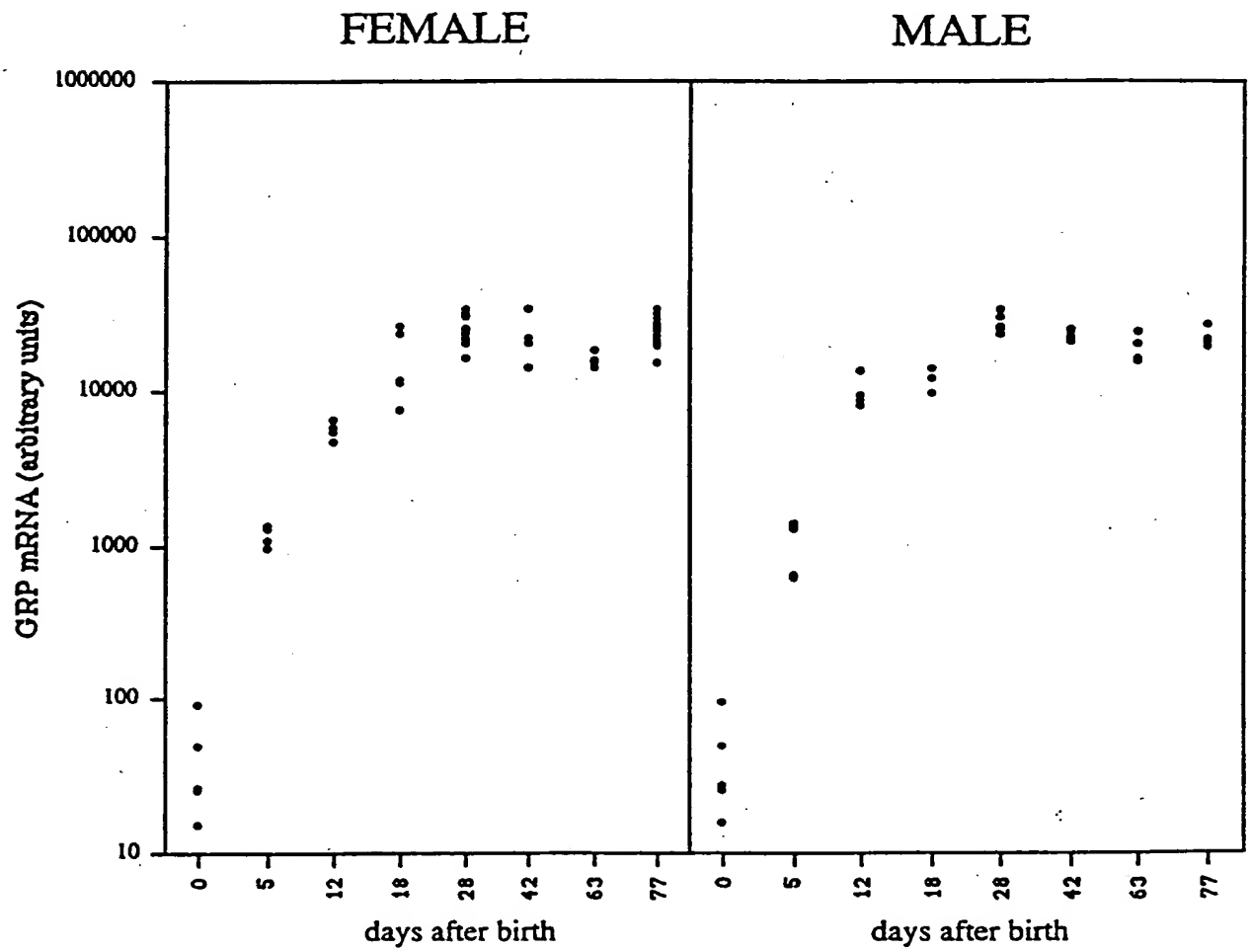


FIG.1

2. Expression of SMR1 encoding during the post-natal development of rat SMG.

We used a RNase protection assay, based on the protection of the 234 bases-long fragment. To correct for loss of material during the numerous steps of this technic, a sequence corresponding to the 5' end of the actin mRNA sequence (protected fragment of 124 bases) was also used. The results were first expressed as the ratio between SMR1 encoding gene and actin signals obtained after separation of the protected probes on a sequencing gel and quantification by phosphorimager. These results are independent of the amount of RNA used in the assay and are directly comparable from one experiment to another. In a second step, these results were corrected for the decrease of actin expression, as previously determined.

As seen in figure 2, SMR1 mRNA was detected at birth but at a very low level. It is thereafter accumulated at the same rate in male and female rats before the beginning of sexual maturation. However, the increase rate for SMR1 mRNA between 0 and 5 days is not so high as GRP mRNA. In female rats, a plateau seems to be reached between 18 days and 6 weeks.

In males, a second raise of SMR1 gene expression is seen by 4 weeks on and significative sex-linked difference ($p<0.01$) of expression is observed from 4 weeks on. Expression increases in male rats about 10-200 fold between 4 and 11 weeks. This period corresponds to the period of puberty, where circulating androgens were shown to rise. Large variations of expression among individues at 4 weeks are compatible with differences in the timing of sexual maturation. In contrast, expression levels in adult male rats do not show large variations.

After the period of the plateau, SMR1 mRNA levels significantly decrease in females. In some adult females, SMR1 expression is less than at day 5.

In situ hybridization using either digoxigenin or radio-labeled SMR1 cDNA probes reveal the presence of SMR1 mRNA in a few cells in the glands of neonates. The number of these positive cells increases during the time course of differentiation. In 12 days, old rats, some highly labeled cells are observed besides a relatively high number of moderately or low expressing cells. The number of highly expressing cells increase in 18-day- and 4-week-old female rats, maximal in some 6-week-old females. In contrast, the number of labeled cells decrease in young adult females, where some highly labeled cells and some moderately labeled cells are observed inside a large majority of negative cells. Long exposure times reveal patches of high labeling. Positive cells are the most often either isolated in an acinus or grouped by two cells. During post-natal development of the male rat SMG, positive cells both increase in number and intensity. For 6 weeks on all acinar cells in males are seen as positive.

Variations of VCSA1 mRNA accumulation during post-natal development of rat SMG

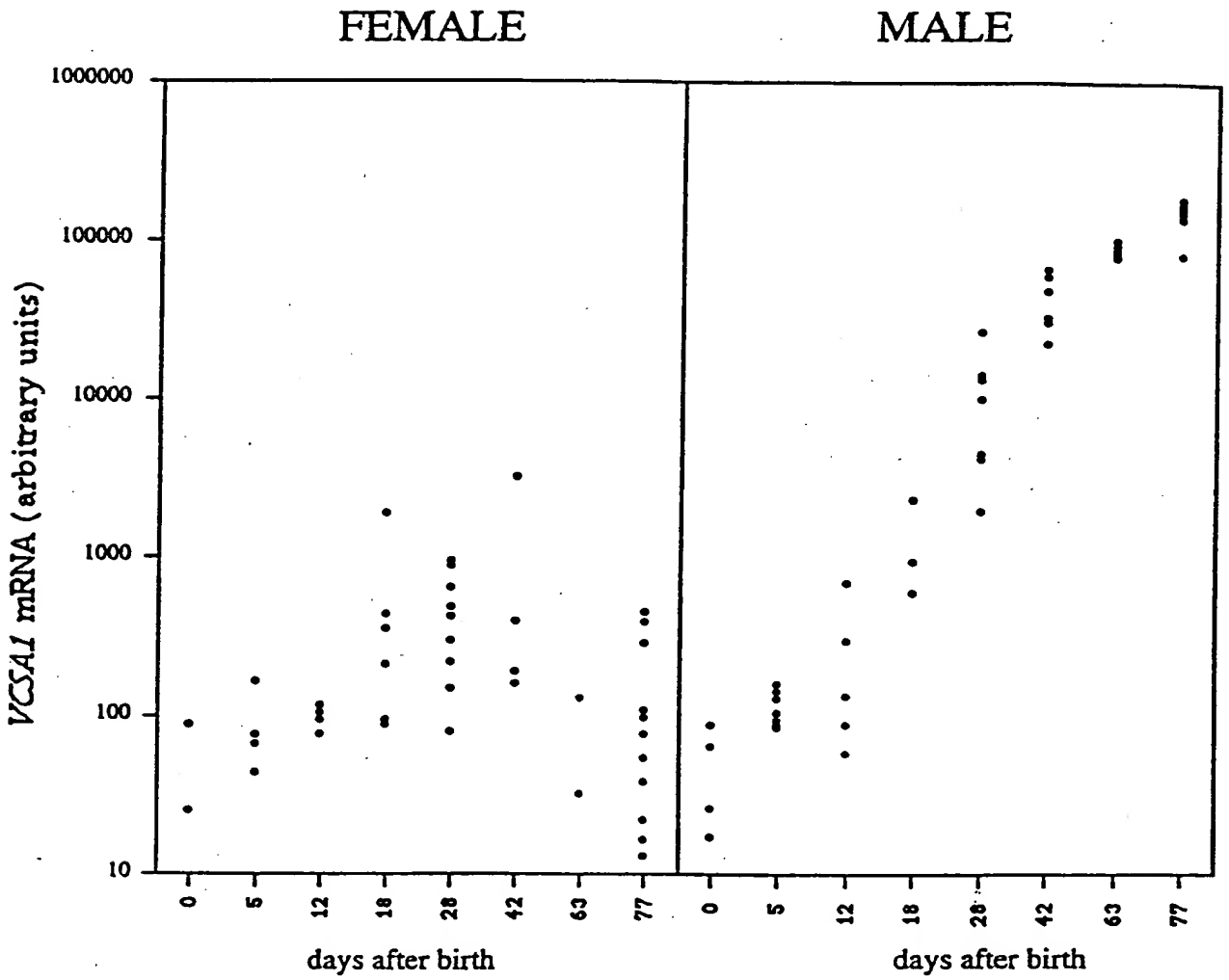


FIG. 2

Conclusion

These results show that SMR1 encoding gene expression is correlated with acinar differentiation on the one hand and with androgen increase during puberty in males on the other hand.

C. *In vivo* biosynthesis of SMR1-related mature peptides

Materials and methods

Radioimmunoassay of SMR1-derived pentapeptide

Peptide

The peptides corresponding to the sequence Glp-His-Asn-Pro-Arg and Gln-Asn-Pro-Arg were synthetized by the Laboratoire de Chimie Organique, Institut Pasteur, Paris, France.

Antiserum

Antiserum L 247 was raised in rabbits immunized against [Gln^1]-His-Asn-Pro-Arg and [Glp^1]-His-Asn-Pro-Arg pentapeptides conjugated to egg albumin (Calbiochem) using the benzidine-bis-diazoted procedure (four peptide residues/molecule of ovalbumin).

Radio-iodinated label

The peptide Glp-His-Asn-Pro-Arg was labeled according to the method of Greenwood et al. using 1 mCiNa^{125}I (Amersham), 0.8 µg (1nmol) pentapeptide and 15 µg chloramin T (Fluka) in 50 µgl sodium borate, pH 8. After a 2-min reaction, the mixture was chromatographed by GFO5 gel filtration (Pharmacia-LKB) to isolate the ^{125}I-labelled pentapeptide (monomer form) and was subsequently chromatographed on reverse-phase porapak Q (Waters-Millipore) to elute the mono-iodinated-labeled pentapeptide. The specific radioactivity, corresponding to one atom or radioiodine/peptide molecule, was estimated as 1500Ci/mmol.

Competitive radioimmunoassay

The liquid-phase RIA was performed in 0.2 M Tris/HCl, pH 8.5 containing 0.25 % bovine serum albumin (Fr 5 Miles) 0.1 % Triton X-100 (Sigma), 100 KIU (kallikrein international unit)/ml Trasylol (a trypsin and kallikrein inhibitor ; Bayer) and 0.1 g/l NaN_3. Standard or sample (0.1 ml) diluted anti-pentapeptide serum (0.1 ml) and ^{125}I-labeled pentapeptide (15 x 10^3 dpm, 0.1 ml) were incubated overnight at 4°C. Bound and free fractions were separated by propanol precipitation (10 µl normal rabbit serum and 1 ml ice-cold 1-propanol) and the radioactivity of the precipitate was determined using a gamma counter (LKB-Pharmacia).

Under these conditions, at a final dilution of 1:600, the anti-pentapeptide serum bound 30 % of the ^{125}I-labeled pentapeptide. 50 % displacement of the ^{125}I-labeled pentapeptide was obtained with 570 fmol pentapeptide standard ([Glp^1]pentapeptide or [Gln^1]pentapeptide). The detection limit (80 % binding) was 106 fmol standard peptide. The inter-assay variation coefficient was 12 %, n=24. The cross-reactivity of the antiserum with tetrapeptide sequence (Glp-His-Asn-Pro) and thyrotropin-releasing hormone (Glp-His-Asn-Pro) was less than 0.01 %.

Sample preparation

Adult male Wistar rats, purchased from Iffa-Credo (France) were kept at 2-5 animals/cage under controlled lighting and temperature with free access to food and water.

Tissues

The rats were killed by decapitation and the different organs were rapidly removed. Tissues were subsequently homogenized at 4°C in ten volumes of 0.1 M HCl with a Potter homogenizer (Heidolph, bioblock). This acidic medium is assumed to inhibit proteolytic activities. Homogenates were centrifuged for 30 min at 4°C and 15000g. The supernatants were divided into aliquots which were stored at -80°C until the methanol-extraction procedure was performed.

The protein concentration was determined by the Bradford method.

Saliva and blood plasma

Adult male Wistar rats (340-380 g, 12-week-old animals) were anesthetized with intraperitoneally injected sodium pentobarbital (45 mg/kg). A silastic catheter was implanted into the right external jugular vein down to the atrium and, on one side,

the main submandibular duct was cleared and incised without bleeding. Viscous saliva samples were collected for 30 min before and after secretagogue administration. Blood samples (0.5 ml in a heparinized syringue, 25 IU) were withdrawn before and 5, 15, 30, 45 and 60 min after the drug treatment. The saliva and blood samples were collected into previously cooled tubes, containing Trasylol, a trypsin and kallikrein inhibitor (Bayer, France ; 1000 KIU/ml final concentration). Blood samples were centrifuged for 1.5 min at 4000 g and 4°C. Plasma fractions and saliva samples were stored at -80°C until the methanol-extraction procedure was performed.

Extraction of low-molecular-mass components

Aliquots of HCl extracts (one volume) were neutralized at 0°C (one volume cold 0.5 M Tris/HCl, pH 8.5) and rapidly transferred on ice to microsorb tubes (Nunc) which contained four volumes of methanol (67 % final concentration). Saliva and blood samples (one volume) were directly transferred into previously cooled tubes containing two volumes of methanol.

The methanol mixtures were quickly vortexed, to rapidly inactivate degrading enzymes, and centrifuged for 40 min at 4°C and 15000 g. Methanol was removed from the supernatant in a vacuum centrifuge (Speed-vac, Savant). The aqueous phase was frozen and stored at -80°C until tested for the pentapeptide-immunoreactive content before and after chromatographic separations. The recovery of the marker pentapeptide added to the homogenate was 81 %.

Results

Initial study revealed a very high concentration of immunologically detected pentapeptide in the 10-week-old male rat SMG, namely 462 ±81 pmol/mg protein, n=3. To determine whether the expression of pentapeptide-immunoreactive material is tissue specific, we assayed various extracted organs from male rats. The peptide-detection experiments were performed on HCl-homogenized tissues after a methanol-extraction procedure to assess only low-molecular-mass immunoreactive components.

As shown in figure 3A, the prostate is the only organ among those tested, where pentapeptide immunoreactivity was significantly detected : 1.2±0.2 pmol/mg protein. However, the prostate immunoreactivity was ≥ two orders of magnitude less abundant than in the SMG of male rats. The pentapeptide-immunoreactive contents in other organs, including parotid and sublingual glands, were at or below the sensitivity limit of the RIA (≤ 0.3 pmol/mg protein).

Since it has been shown that SMR1 gene expression, in the SMG is regulated by androgens, which are present in rats from the fourth postnatal week onwards, it was of interest to determine the pentapeptide-immunoreactive content in the male rat SMG of different postnatal ages. In related experiments, using 14-week-old male and female rats, the effect of sexual difference was also assessed.

As shown in figure 3B, pentapeptide immunoreactivity was first detected in the male rat SMG at six weeks postnatal age (33 ± 3 pmol/mg protein) and rose rapidly to attain maximal levels at ten weeks. No penta-related peptide was detected in the SMG of 4-week-old males (≤ 0.2 pmol/mg protein).

Levels of the SMG pentapeptide imunoreactivity were much higher in males than in females. For instance, at 14 weeks, the levels were 69 times more elevated in male rats (475 ± 44 pmol/mg protein) than in female rats (6.9 ± 0.8 pmol/ protein ; figure 3B).

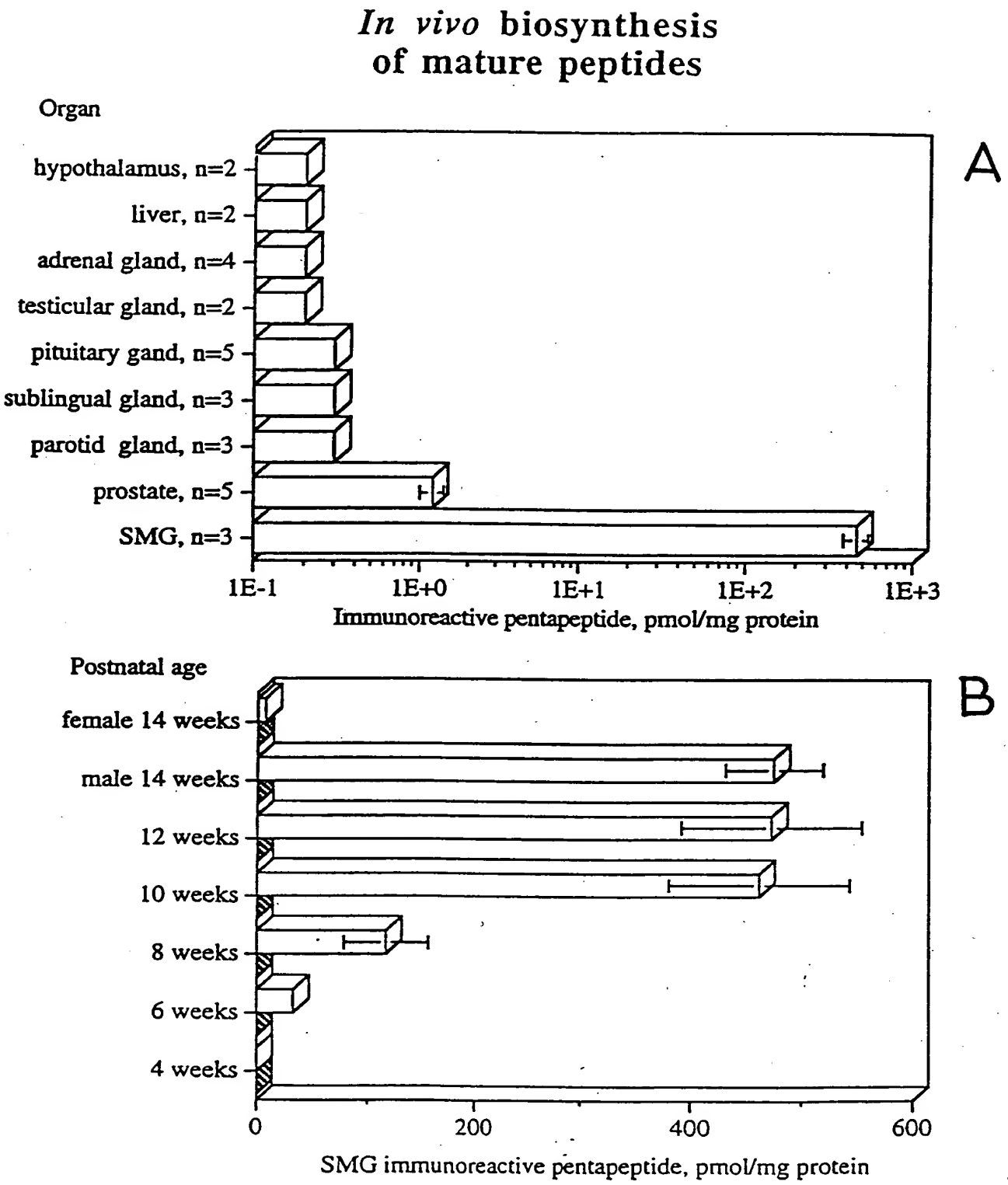

In vivo biosynthesis
of mature peptides
Organ
hypothalamus, n=2
liver, n=2
adrenal gland, n=4
testicular gland, n=2
pituitary gand, n=5
sublingual gland, n=3
parotid gland, n=3
prostate, n=5
SMG, n=3
1E-1
1E+0
1E+1
1E+2
1E+3
Immunoreactive pentapeptide, pmol/mg protein
A
Postnatal age
female 14 weeks
male 14 weeks
12 weeks
10 weeks
8 weeks
6 weeks
4 weeks
0
200
400
600
SMG immunoreactive pentapeptide, pmol/mg protein
B

FIG.3

Conclusion

This assay of SMR1-related peptides shows, on the one hand, that biosynthesis of these peptides strongly increases after puberty and, on the other hand, that the concentration of SMR1-related peptides in SMG is higher in males than in females.

D. Effects of hypophysectomy and gonadectomy on submandibular gland mature peptide levels

Materials and methods

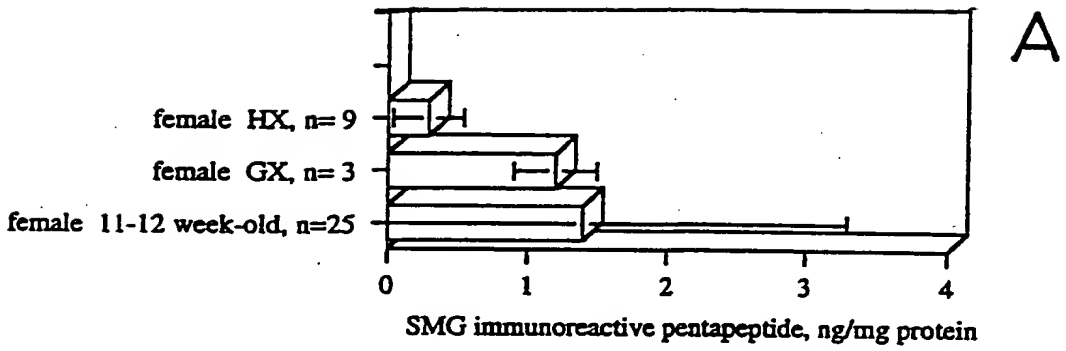
The specific immunoreactivities were measured as described in section C.

A first experiment was carried out on 11-week-old rats which were hypophysectomized 10 days before being killed. A second experiment was carried out on gonadectomized rats. The gonads were removed from 4-week-old rats, which were killed 7 weeks later.

Results

The effects of hypophysectomy and gonadectomy on SMG mature peptide levels are shown on figure 4 (HX = hypophysectomy, GX = gonadectomy).

The expression of SMR1-related peptides is significantly dependent upon the presence of testosterone in plasma.


A
female HX, n= 9
female GX, n= 3
female 11-12 week-old, n=25
0
1
2
3
4
SMG immunoreactive pentapeptide, ng/mg protein

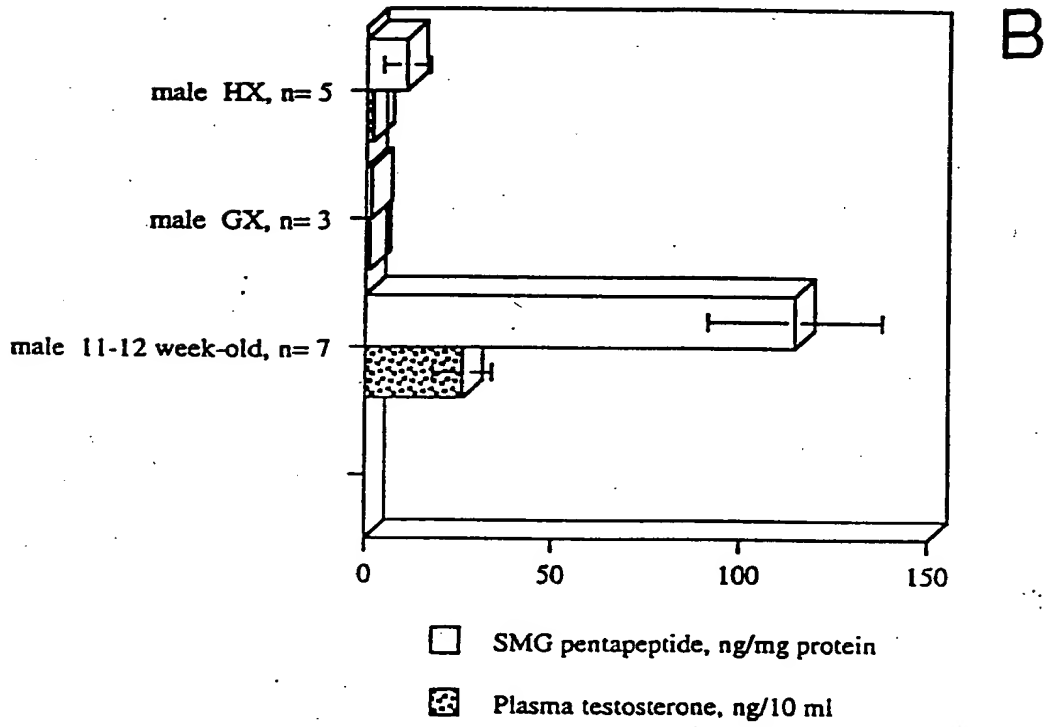

B
male HX, n= 5
male GX, n= 3
male 11-12 week-old, n= 7
0
50
100
150
SMG pentapeptide, ng/mg protein
Plasma testosterone, ng/10 ml

FIG. 4

In light of the above results, it appears that the claimed peptides are involved in biological responses regulated by androgens.
So they can be particularly useful as biological markers for evaluating the androgen or anti-androgen activity of new drugs in preclinical trials.
The claimed peptides are all the more interested that the radioimmunoasssays can be carried out on saliva samples as well, in a simple and rapid procedure.

-o-o-o-o-o-o-o

The undersigned Declarant declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true ; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed this 9 day of June 1997

j. Chrapi